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Award Number: W81XWH-10-1-0917

TITLE: VRP09 Reduction of Corneal Scarring Following Blast and Burn Injuries to Cornea Using siRNAs Targeting TGFb and CTGF

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REPORT DATE: October 201G

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE						Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 201G			2. REPORT TYPE Annual			3. DATES COVERED 27 September 201F – 26 September 201G	
4. TITLE AND SUBTITLE VRP09 Reduction of Corneal Scarring Following Blast and Burn Injuries to Cornea Using siRNAs Targeting TGFb and CTGF						5a. CONTRACT NUMBER	
						5b. GRANT NUMBER W81XWH-10-1-0917	
						5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Gregory Schultz E-Mail: schultzg@ufi.edu						5d. PROJECT NUMBER	
						5e. TASK NUMBER	
						5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Florida Gainesville, Florida 32611-5500						8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012						10. SPONSOR/MONITOR'S ACRONYM(S)	
						11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited							
13. SUPPLEMENTARY NOTES							
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15. SUBJECT TERMS None provided.							
16. SECURITY CLASSIFICATION OF:				17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON	
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	USAMRMC				
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Blast and burn injuries to the eye caused by explosions during combat operations or terrorist attacks are devastating injuries, and in eyes that can be saved, the major causes of vision impairment are excessive corneal scarring and neovascularization. Unfortunately, no approved drugs have been shown to improve vision outcome in eyes with these types of corneal injuries. However, decades of clinical experience and laboratory research have shown that the key to improving vision outcome is to improve the quality of corneal wound healing.^{(1), (2), (3)} Our overall objective is to develop new drugs that use the emerging technology of RNA-interference (RNAi) to reduce vision impairment following corneal injuries by reducing expression of genes that stimulate formation of corneal scar (corneal haze).⁽⁴⁾ We reported previously that corneal scarring is primarily up-regulated by the actions of transforming growth factor beta (TGFb), which stimulates corneal cells by binding to the TGFb type II receptor (TGFbRII) and inducing expression of connective tissue growth factor (CTGF).⁽⁵⁾ CTGF then directly up-regulates synthesis of collagen scar and induces transformation of fibroblasts into myofibroblasts. Our approach is to design and test small interfering RNAs (siRNA) that will selectively reduce the level of expression of these three key proteins that stimulate corneal scar formation, and thereby, reduce vision loss. We focused on siRNAs because they are the most potent and selective of all gene-targeted, oligonucleotide-based drug approaches (better than ribozymes, antisense oligonucleotides (ASO), or microRNAs).⁽⁴⁾ We will accomplish this objective in three specific aims. First, we will design and test siRNAs that selectively target the mRNAs of each of the three target genes, TGFb, TGFbRII and CTGF, using cultures of rabbit corneal fibroblasts (RCF). We will then test the optimal siRNA for each gene when formulated into double and triple combinations using cultures of RCF to obtain the maximum knock-down of collagen synthesis. Second, we will test the two most effective siRNA combinations for reduction of corneal scarring (haze) using the rabbit models of blast and burn corneal injuries. Third, we will compare the effectiveness of siRNAs oligonucleotides and AAV-vectored siRNAs in rabbit models of blast and burn corneal injuries for reduction of corneal scarring (haze).

BODY

We accomplished all the primary objectives for the second year of this project, and we are on schedule to begin the next phase of developing an effective antiscarring drug therapy for corneal blast and burn injuries. Starting with *in vitro* results and by using computational analysis, we generated two siRNA triple combinations - an "Effective" with optimal synergy and an "Ineffective" with poor synergy among its individual sequences. The efficient combination led to significant reduction in the steady state levels of the target growth factors and of the downstream mediators like Collagen-I and SMA and outperformed the ineffective combination. Employing *ex vivo* organ culture and *in vivo* animal testing, we were able to establish a method to deliver the triple combination using nanoparticles. The result from this study paves the way towards developing a multi target approach for the reduction of corneal haze and perhaps of other scarring processes affecting the skin.

Objective 1a. Test the previously identified efficient triple combination in low passage cultures of rabbit corneal fibroblasts for consistency in giving high knockdown percentages of the target growth factors and their downstream mediators like collagen and alpha Smooth Muscle Actin.

Low passage number rabbit corneal fibroblast cultures were grown to near confluency in T-75 culture flasks in DMEM supplemented with 10% newborn calf serum (NCS). The cells were harvested with trypsin/EDTA solution and 100 ul of resuspended cell suspension containing $\sim 10^5$ were seeded into cells 96 well culture plates in DMEM medium containing 10% NCS. When the cells reached initial confluency, the medium was removed and replaced with serum-free DMEM. After 48 hours of culture, estradiol and TGFB1 was added to the medium. After 24 hours of incubation, a second dose of estradiol and TGFb1 was given to the cells and the cells were transfected with the triple siRNA combination using the optimized transfection reagent and after 6 hours of incubation with the siRNA/transfection reagent, the medium was removed and replaced with DMEM. After a further 24 hours of incubation a third dose of estradiol and TGFB1 was given. After 24 hours, RNA was collected. cDNA library was generated and qRT PCR was performed to analyze the levels of target growth factors and downstream mediators.

Figure 1. Knockdown of target growth factors by Synergistic Triple combination (T1R2C1). Cultures of Rabbit corneal fibroblasts stimulated with TGFB1 and Estradiol were transfected with the triple siRNA combination (15nM, 30nM, 60nM and 90nM) using Mirus transfection reagent. The RNA samples were collected 48 hours after transfection and were analyzed using qRT PCR. On a similar experiment, cells were dosed with increasing concentrations of siRNA triple combination (90nM to 180nM) and cell viability was assessed using the MTS assay. The knockdown percentages of a) TGFB1, b) TGFB2 and c) CTGF are plotted for the identified synergistic triple combination (T1R2C1). All expressions were normalized to 18S rRNA. The knockdown percentages were calculated with respect to a scrambled siRNA control. d) The percentage of viable rabbit corneal epithelial cells after treating with increasing concentrations of the synergistic triple combination (T1R2C1).

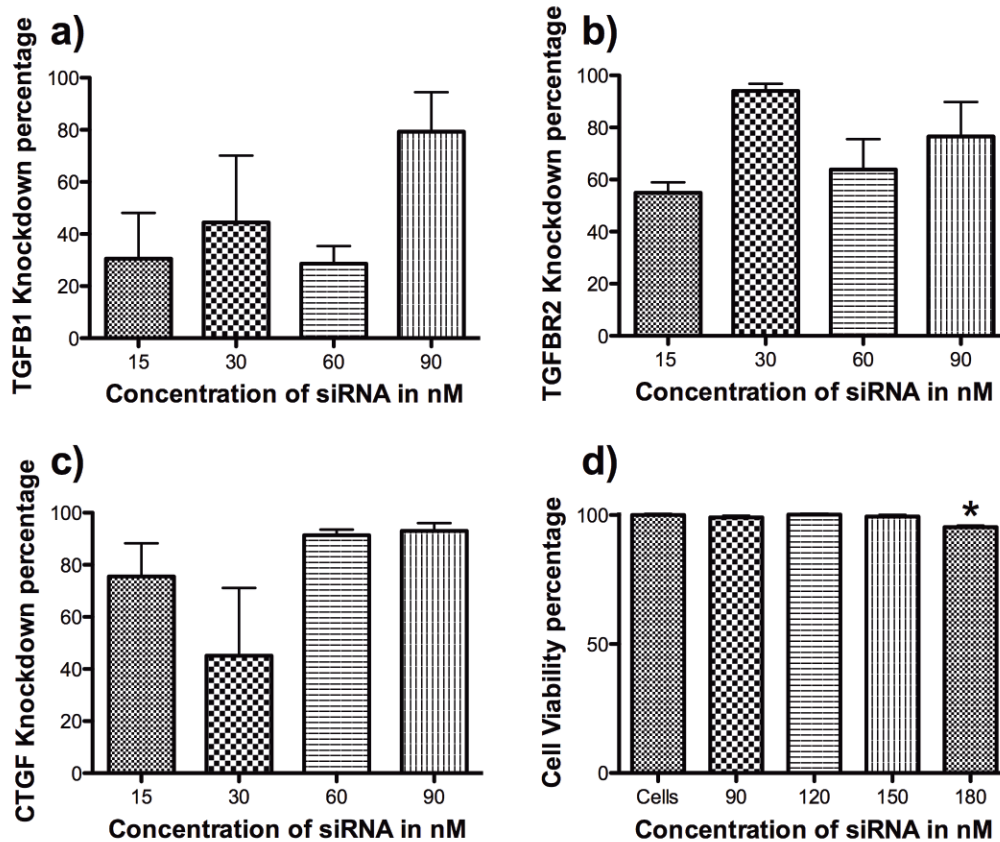
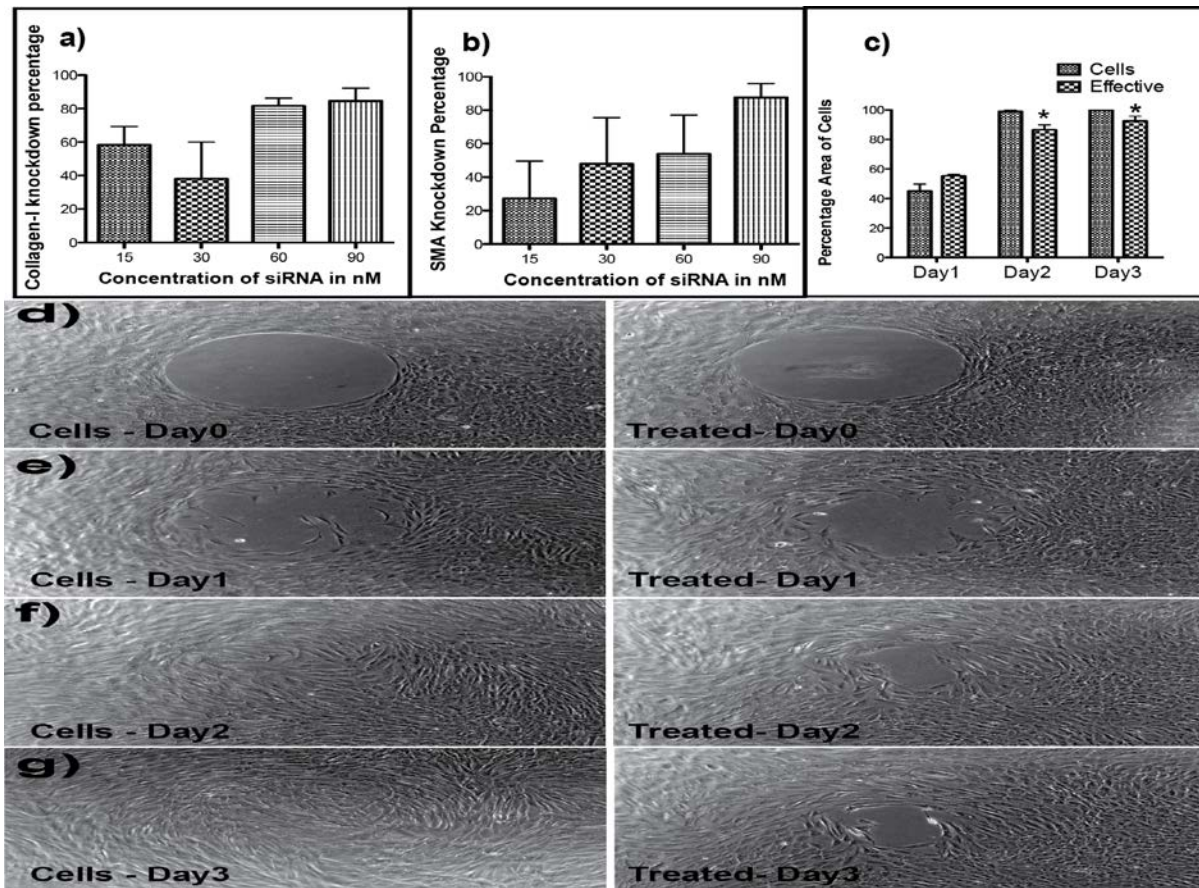


Figure 2. Reduction of Downstream mediators by the Synergistic Triple combination (T1R2C1). Cultures of Rabbit corneal fibroblasts stimulated with TGFB1 and Estradiol were transfected with the triple siRNA combination (90nM) using Mirus transfection reagent. The RNA samples were collected 48 hours after transfection and were analyzed using qRT PCR. The RNA level reduction percentages of a) Collagen-I and b) SMA are plotted for the identified synergistic triple combination (T1R2C1). All expression levels were normalized to 18S rRNA. The knockdown percentages were calculated with respect to a scrambled siRNA control. For the migration assay, a circular region in the middle of the well was removed using a gel removal solution provided with the Radius® cell migration kit. Panels d) – g) show migration assay results comparing cells alone and synergistic triple combination (T1R2C1) treatment wells. c) gives the percentage area of cells from the migration assays calculated through image analysis from 3 replicate wells.

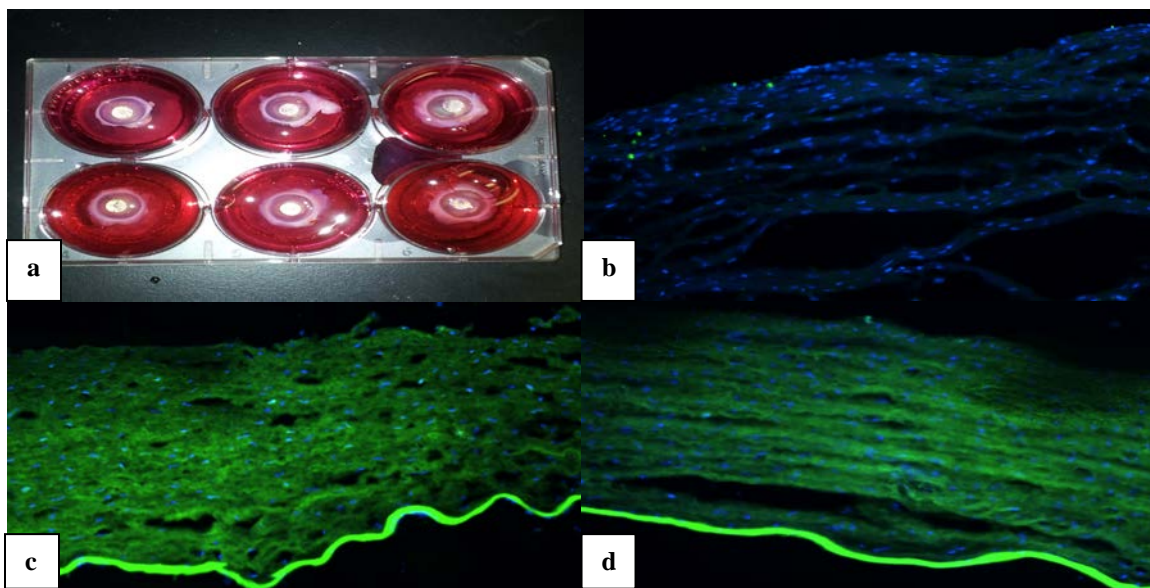


Objective 1b. Assess the effectiveness of nanoparticles complexed with siRNAs to deliver the siRNAs to corneal cells in rabbit eyes.

We had previously assess the effectiveness of iontophoretic delivery of small oligonucleotide sequences (antisense oligonucleotides, ASOs) into rabbit corneal cells and found that technique was very effective in delivering ASOs into the stroma and even into the endothelial cell layer. However, that technique requires the use of a specialized iontophoresis cell that attaches to the cornea surface and a low voltage DC power supply to deliver the electrical current that moves the charged ASOs into the cornea.

To simplify the delivery approach, we assessed the effect of nanoparticles complexed with siRNAs for delivery to rabbit corneal cells. As shown in Figure 3, we generated nanocarrier particles complexed with siGLO which is a fluorescein-labeled siRNA that is a surrogate for our siRNAs targeting the three target genes. The siGLO complexed to nanocarrier particles that we generated with a commercially available kit of biodegradable polymers were tested in vitro using fresh rabbit globes that were ablated by excimer laser to simulate a corneal blast or burn wound. The basic in vitro test model consisted of topically applying the siGLO reporter oligo complexed to nanocarrier particles for 1 minute to the ablated area with the aid of a 14 mm vacuum trephine collar attached to the cornea. The corneas were placed in 6-well culture plates and maintained for several hours to assess uptake of the fluorescein-labeled siRNA nanocarrier particles (panel A). As seen in panel B, the uptake of siGLO when applied to the corneas without nanocarrier particles was very minimal and typically limited to the upper 1/3 of the corneal stroma. In marked contrast, application of the siGLO complexed to nanocarrier particles (panel C) after brief pretreatment of the corneas with a very dilute EDTA solution to increase permeability of the epithelial cell layer, or in panel D without EDTA pretreatment showed extensive fluorescence even to the level of Descemet's membrane, demonstrating penetration of the siGLO throughout all layers of the rabbit corneas. These results indicate that topical delivery of siRNAs to corneas wounded by blast injuries can be accomplished rapidly and with essentially no specialized instruments when the siRNAs are complexed with nanocarrier particles. These results will be a great advantage in the next series of experiments when we treat rabbit corneas following excimer laser ablation with the triple combination of siRNAs.

Figure 3. Nanoparticles deliver siRNA to all layers of the cornea in organ-culture and rabbits. Whole rabbit globes were ablated to 125 microns using an excimer laser and treated with 3 doses of fluorescently labeled scrambled siRNA complexed with a nanoparticle for 1 minute. a) shows the *ex vivo* organ culture of the excised rabbit corneas. Delivery of fluorescently labeled siRNA to different corneal layers is compared between – b) siRNA alone c) siRNA using nanoparticles d) magnified image of cornea delivered with fluorescently labeled siRNA sequence. The blue color shows the cell nuclei, which were stained with DAPI, and the green shows the fluorescence of the delivered scrambled siRNA.



Objective 1c – To confirm previously reported significant reduction of the downstream mediator of scarring, smooth muscle actin (SMA), by using immunohistochemistry. Also, compare the efficacy of the efficient triple combination to an inefficient triple combination with poor synergism between the siRNA sequences to knockdown collagen.

Low passage number rabbit corneal fibroblast cultures were grown to near confluency in T-75 culture flasks in DMEM supplemented with 10% newborn calf serum (NCS). The cells were harvested with trypsin/EDTA solution and 100 ul of resuspended cell suspension containing $\sim 10^5$ were seeded into cells 96 well culture plates in DMEM medium containing 10% NCS. When the cells reached initial confluency, the medium was removed and replaced with serum-free DMEM. After 48 hours of culture, estradiol and TGFB1 was added to the medium. After 24 hours of incubation, a second dose of estradiol and TGFb1 was given to the cells and the cells were transfected with the triple siRNA combination using the optimized transfection reagent and after 6 hours of incubation with the siRNA/transfection reagent, the medium was removed and replaced with DMEM. After a further 24 hours of incubation a third dose of estradiol and TGFB1 was given. After 24 hours, the cells were fixed in 4% paraformaldehyde for 10 minutes. They were then washed in PBS and treated with cold methanol for 15 minutes at -20C. They were washed again in PBS and blocked in goat serum for 1 hour. The samples were incubated with an SMA antibody conjugated with Cy3 for 1 hour. The cells were wash again in PBS before mounting them with DAPI. They were imaged using a confocal microscope for taking image.

Figure 4. The Synergistic triple combination (T1R2C1) is more effective than the Non-synergistic triple combination (T2R1C2) in blocking downstream mediators. Cultures of Rabbit corneal fibroblasts stimulated with TGFB1 and Estradiol were transfected with synergistic (T1R2C1) and non-synergistic (T2R1C2) triple siRNA combination (90nM) using Mirus transfection reagent. The RNA samples were collected 48 hours after transfection and were analyzed using qRT PCR. a) The RNA level reduction percentages of five genes (target and downstream) are compared between the synergistic (T1R2C1) and the non-synergistic triple combination (T2R1C2). All expression levels were normalized to 18S rRNA. The knockdown percentages were calculated with respect to a scrambled siRNA control. To stain for SMA, forty-eight hours after transfection, the cells are fixed in 4% paraformaldehyde, blocked in goat serum and then incubated with cy3 labeled SMA antibody. Images of SMA protein immunohistostaining at 20X are shown for: b) synergistic triple combination (T1R2C1), c) non-synergistic triple combination (T2R1C2) and d) cells alone. e) gives the percentage reduction of SMA staining calculated through image analysis from 4 different areas in 3 replicate wells. ($p < 0.05$)

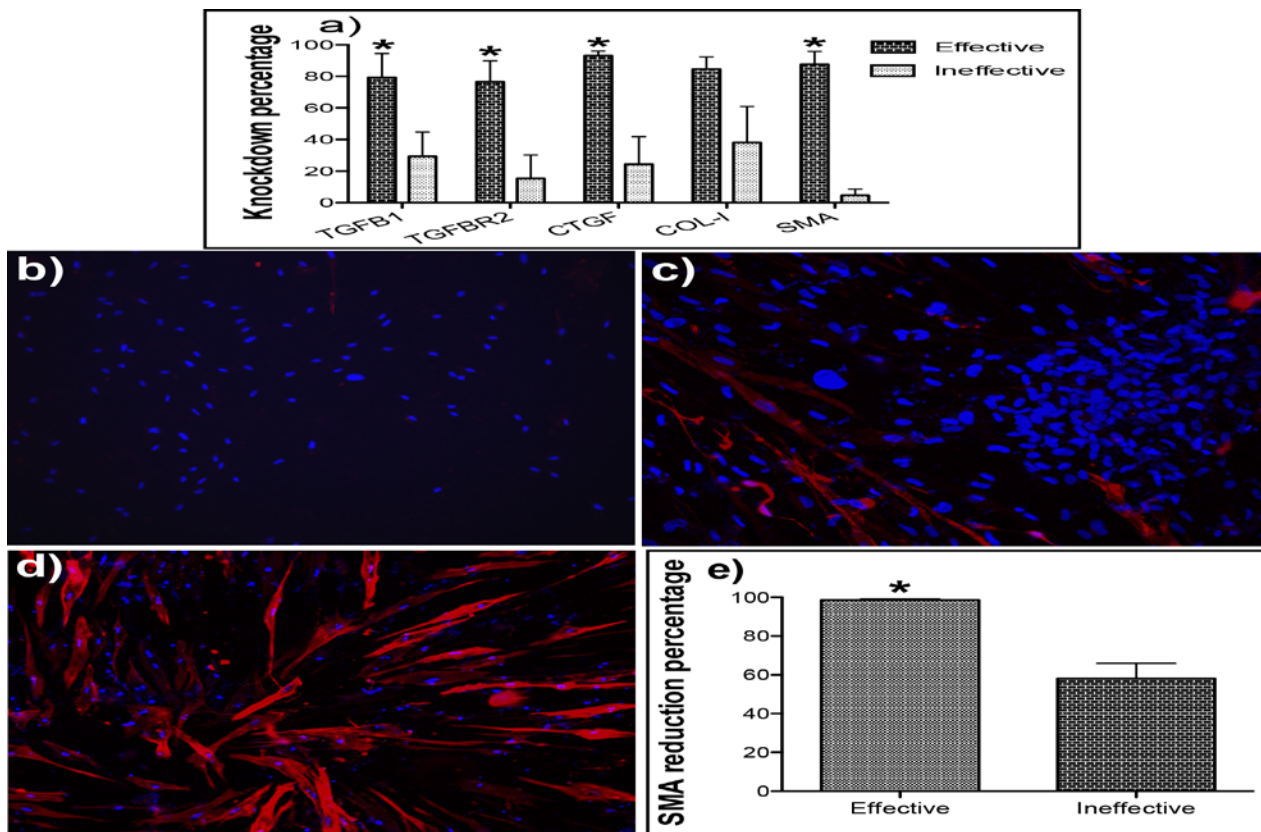
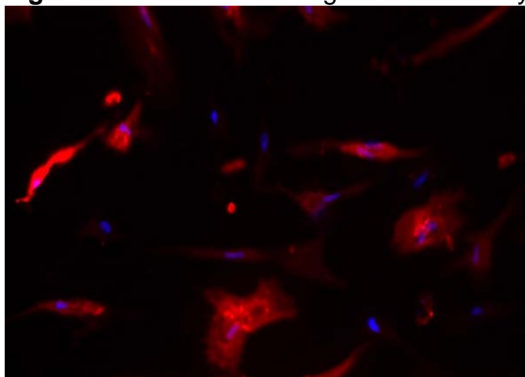


Image analysis procedure

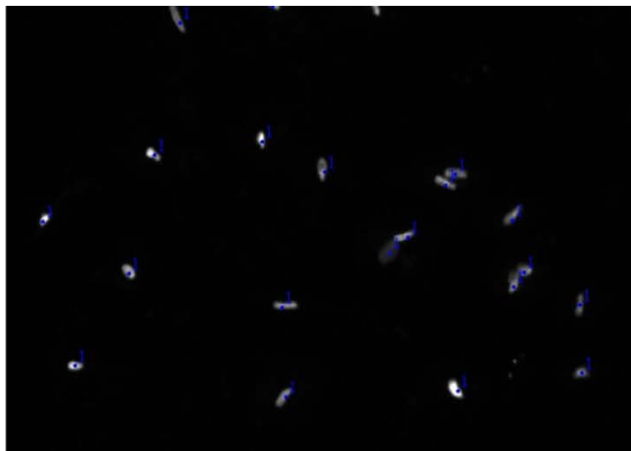
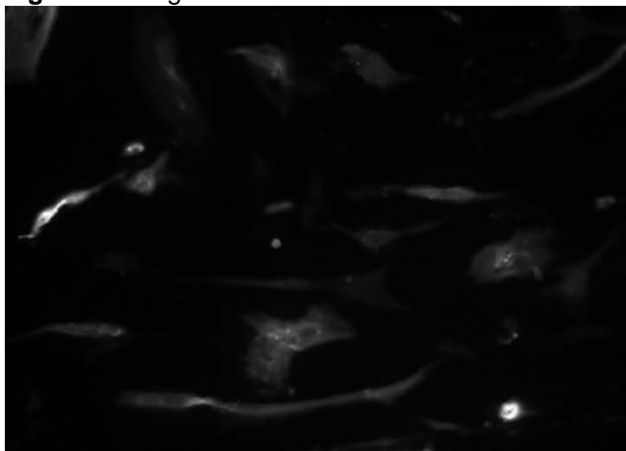
Step1. The images are initially opened using the software ImageJ.

Figure 5. 20x confocal image that was analyzed for levels of alpha smooth muscle actin staining.



Step2. The image was split into 2 channels – red and blue. SMA was detected by the red channel and the blue channel detected by the DAPI stained nuclei. The number of cells is counted using the cell counter tool.

Figure 6. Image viewed under red and blue channels.



Step3. A color threshold was applied so that only the SMA stained areas are detected. Finally, the percent area stained by SMA was measured and expressed in terms of the number of cells.

Figure 7. Application of a color threshold to detect the SMA stained areas.



	Area Mean	% Area
Total	344064	100
SMA Stained	23,114	6.72

The graph comparing the reduction of SMA in cells treated with synergistic and non-synergistic siRNA triple combination is given in Figure 4-e. It comports with the previously obtained RNA level knockdown measured using qRT-PCR.

Objective 1d– To study the change in expression of CTGF and TGFB1 after excimer ablation in rabbit corneas after ablation at different time points to improve siRNA targeting knowledge.

An excimer laser was used to reproducibly create deep (1/3 the total depth of the cornea), bilateral, PTK ablations (8 mm diameter) to the central cornea of adult NZW rabbits. After ablation, rabbits were euthanized at 2 different time points, day 1 and day 2. A total of 4 rabbits were used for each time point. The cornea was excised and RNA from the different layers (epithelium, stroma and endothelium) were extracted. A cDNA library was generated which was used to probe the expressions of TGFB1 and CTGF. On day 1, the highest expression of CTGF mRNA was in the endothelium, followed by stroma and finally the epithelium. However on Day 2, the stroma had the greatest levels of expression of CTGF. On day1, there was very little expression TGFB1 mRNA throughout all cell layers of the cornea. On Day 2, there was a large increase in the production of TGFB1. The highest expression of TGFB1 mRNA was found in the endothelium followed by stroma and epithelium.

Figure 8. Change in fold CTGF expression at different time points post ablation

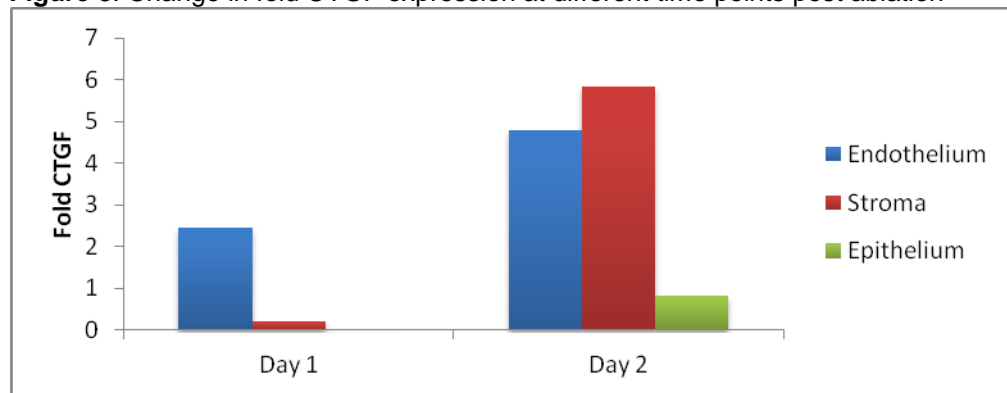
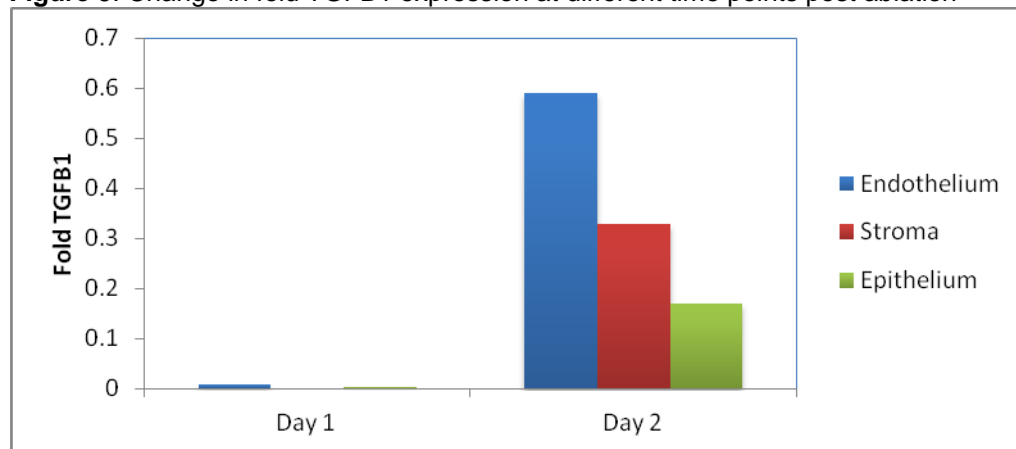


Figure 9. Change in fold TGFB1 expression at different time points post ablation



Objective 1e – To optimize the parameters of the in-vivo confocal microscope using rabbit globes. Commercially available rabbit globes were ordered from Pelfreeze. They were then used to optimize the various parameters of the invivo confocal microscope so that optimized images of the three different layers can be visualized. These settings will be used to analyze of the ability of the triple combination of the siRNAs to reduce haze in rabbit corneas after ablation.

Figure 10. Image of the endothelial layer

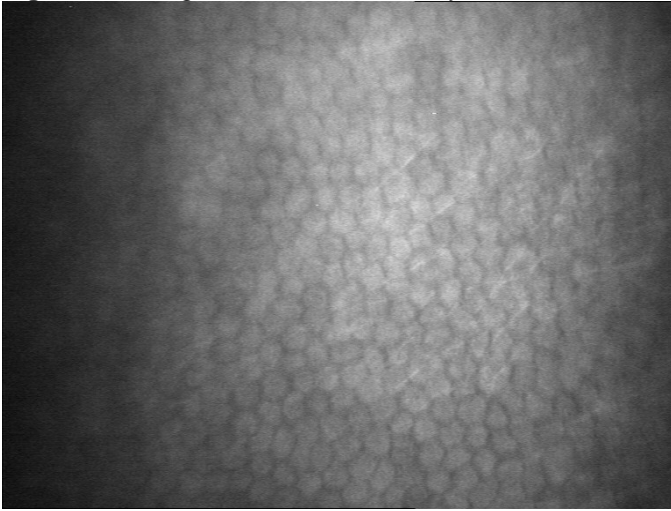
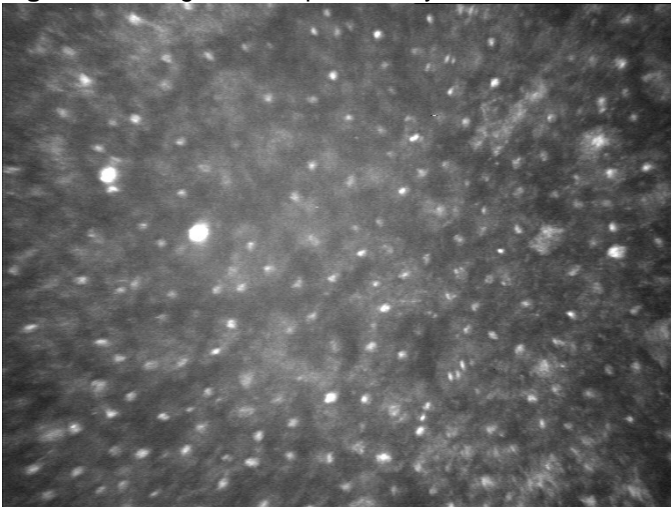


Figure 11. Image of the stromal layer



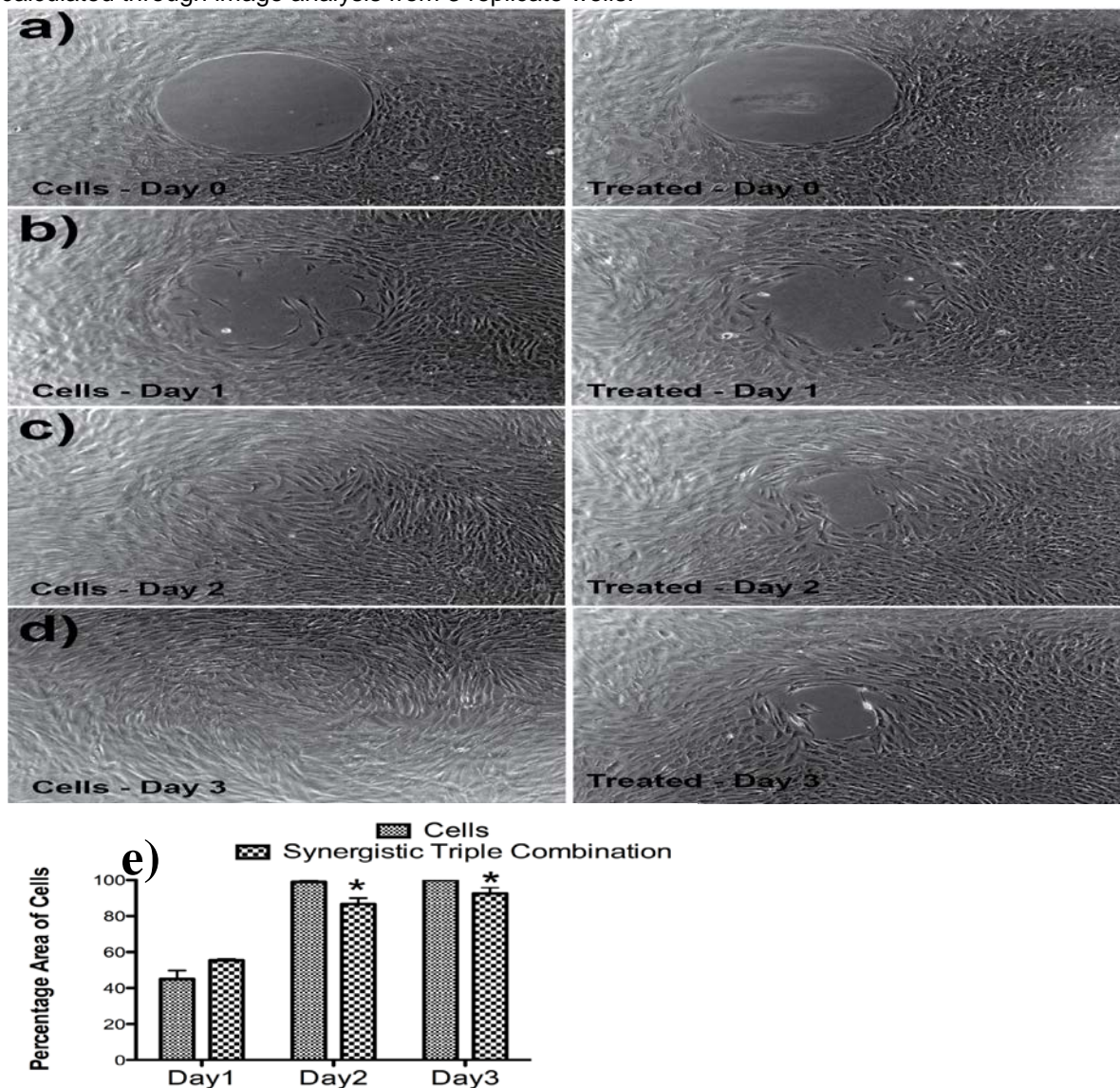
Figure 12. Image of the epithelial layer



Objective 1f – To evaluate the ability of the synergistic siRNA triple combination to retard the migration of corneal fibroblasts cells in culture

Cultures of rabbit corneal fibroblasts (RbCF) were established by outgrowth from rabbit whole eyes, as described previously (Woost, Jumblatt, Eiferman, & Schultz, 1992). Briefly, epithelial and endothelial cells were removed from corneas, the stroma was cut into cubes of approximately 1 mm³, placed in culture medium consisting of equal parts Dulbecco's Modified Eagle Medium (DMEM), with 4.5g/L Glucose and 1g/L L-glutamine. Medium was supplemented with 10% heat-inactivated normal calf serum and 1× antibiotic-antimycotic (Gibco BRL). Cell from cultures between passages 2 and 5 were used for all experiments. To increase the expression of TGFB1, TGFB2 and CTGF, RbCF were place in serum-free media for 48 hours, then the medium was replaced by 8ug/ml of estradiol (Sigma, St. Louis, MO) and TGF-B1 (R&D Systems, Minneapolis, MN) in DMEM. The cells were dosed twice with the estradiol before transfection of the siRNA (Takahashi et al., 1994; Wira, 2002). For the migration assay, Radius™ Cell Migration Plate (Cell biolabs, San Diego, CA) was procured and used according to manufacturer's protocol. Images were taken at 10x magnification and analyzed using Adobe Photoshop.

Figure 13. For the migration assay, a circular region in the middle of the well was removed using a gel removal solution provided with the Radius® cell migration kit. Panels a) – d) show migration assay results comparing cells alone and synergistic triple combination (T1R2C1) treatment wells. e) gives the percentage area of cells from the migration assays calculated through image analysis from 3 replicate wells.

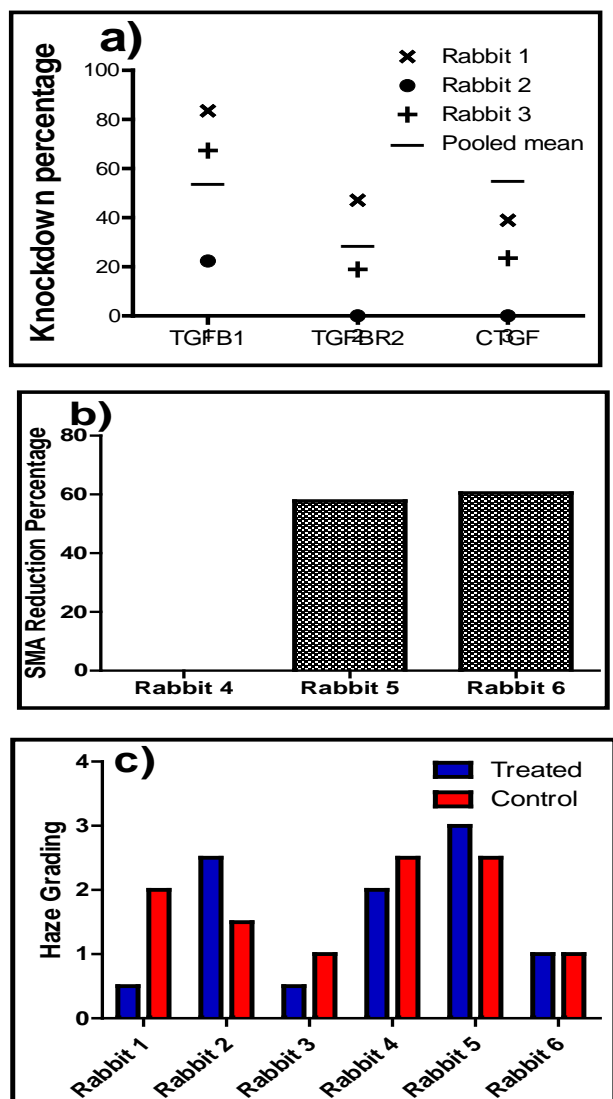


Apart from reducing the above downstream mediators, the synergistic triple combination (T1R2C1) was also able to significantly inhibit the migration of cells within two days post transfection. The percentage area of cells which was calculated digitally from the three replicates was used as a measure of migration (Figure 3-c)

Objective 1g - Observe and evaluate the effects of a topical application of the previously optimized siRNA-nanocarrier complex post wounding in a rabbit model

Adult New Zealand Rabbits free of disease were used and treated according to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Excimer ablation and collection of corneas was performed as previously described (Netto et al., 2006). Briefly, rabbits were anesthetized with isoflurane inhalation, and proparacaine eye drops provided topical anesthesia. Laser ablations were performed to both eyes of each rabbit with a Summit SVS excimer laser that is committed to animal vision research. Using the laser in phototherapeutic keratectomy mode, the central 6 mm diameter area of the cornea was be ablated at a dose of 160mJ/cm² to an initial depth of 80 microns to remove the epithelium and then the final 45 microns were ablated by placing a mesh over the cornea to make an uneven ablation. The eyes were pretreated with 50uM EDTA for 10 minutes. A total of 150ul of the nanoparticle complexed with the siRNA triple combination was added to the right eye while the left eye was treated with the vehicle control and was considered as a paired negative control. The eyes were held open for 3 minutes to allow the nanoparticle to penetrate the stroma before being disturbed. No postoperative topical steroid was used to ensure that the wound healing process is not altered with anti-inflammatory agents. One and fifteen days after the treatment, corneas were collected, homogenized in a pestle with liquid nitrogen and then transferred to TRIzol. The RNA was then extracted using a hybrid RNA extraction protocol with RNeasy spin columns (Rodriguez-Lanetty, 2007).

Figure 14 - For the in vivo experiment, the corneas of 6 rabbits were unevenly ablated to 125 microns using an excimer laser. The right eye was treated with 150uL of the synergistic triple combination (T1R2C1) complexed with nanoparticles and the left eye received the vehicle control. Three rabbits for each time point were sacrificed and RNA was extracted for analysis by qRT PCR at 1 and 14 days post siRNA treatment. a) gives the RNA level knockdown percentages of the target growth factors at day1 while b) gives the RNA level knockdown percentage of SMA at day15. All expressions were normalized to 18S rRNA and knockdown percentages were calculated with respect to the left eye. The corneal scarring was graded by an ophthalmologist on a scale from 0-4. The scores for both the treated and control eyes are plotted for each rabbit in c)



In the day1 samples, the synergistic triple combination (T1R2C1) gave an average of knockdown of 57% for TGFB1, 25 % for TGFBR2 and 24% for CTGF (Figure1-a). One of the rabbits (rabbit 1) had a maximum knockdown of 80% for TGFB1, 57% for TGFBR2 and 46% for CTGF indicating some the siRNA combination was effectively delivered to the corneal stroma in this animal. In the day15 samples, the synergistic triple combination (T1R2C1) gave an average knockdown of ~40% in the RNA level expression of SMA. Two of the three rabbits show a ~60% reduction in the expression of SMA demonstrating a reduction in the RNA levels of SMA by treatment with synergistic triple combination (T1R2C1) (Figure1-b). All knockdown percentages were calculated with respect to the left eye, which received vehicle control without the siRNA. The haze grading of the rabbits also showed a positive trend. Three out of the six rabbits had a lower haze grading score after a period of two weeks (Figure1-c).

REPORTABLE OUTCOMES

The results of these experiments will be presented as an abstract at the 2013 annual meeting of the Association for Research in Vision and Ophthalmology and at the 2013 annual meeting of the Wound Healing Society. In addition a manuscript is being written for submission to an appropriate research journal like Nature Biotechnology.

CONCLUSIONS

In summary, in this study we have developed a gene therapy treatment to reduce corneal haze by targeting three critical scarring genes: TGFB1, TGFBR2 and CTGF. Starting with *in vitro* results and by using computational analysis we generated an efficient triple siRNA combination with optimal synergy among its individual sequences. Employing a hybrid system of *ex vivo* organ culture and *in vivo* animal testing, we were able to establish a method to deliver the triple combination using nanoparticles. The result from this study paves the way towards developing a multi target approach for the reduction of not only corneal haze but also scarring in the entire body.

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APPENDICES

NONE